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Abstract: The tumor microenvironment plays a pivotal role during cancer development and progression. The balance between suppressive and cytotoxic responses of the tumor immune microenvironment has been shown to have a direct effect on the final outcome in various human and experimental tumors. Recently, we demonstrated that the oxygen sensor HIF-prolyl hydroxylase 2 (PHD2) plays a detrimental role in tumor cells, stimulating systemic growth and metastasis in mice. In the current study, we show that the conditional ablation of PHD2 in the hematopoietic system also leads to reduced tumor volume, intriguingly generated by an imbalance between enhanced cell death and improved proliferation of tumor cells. This effect seems to rely on the overall down regulation of pro- as well as anti-tumoral cytokines. Using different genetic approaches, we were able to confine this complex phenotype to the crosstalk of PHD2-deficient myeloid cells and T-lymphocytes. Taken together, our findings reveal a multifaceted role for PHD2 in several hematopoietic lineages during tumor development and might have important implications for the development of tumor therapies in the future. © 2013 Wiley Periodicals, Inc.

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Loss of Prolyl Hydroxylase-2 in Myeloid Cells and T-lymphocytes Impairs Tumor Development

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In the current study, we describe the complex role of PHD2 in tumor associated immune cells in a conditional PHD2-deficient mouse line. We demonstrate reduced tumor volume in these mutant mice as a consequence of opposing changes including the drastic down-regulation of numerous pro- as well as anti-tumoral genes and an imbalance between enhanced cell death and improved proliferation of tumor cells. Using different immune cell specific KO mice, we confined this complex phenotype to the crosstalk of PHD2-deficient myeloid cells and T-lymphocytes.

Abstract

The tumor microenvironment plays a pivotal role during cancer development and progression. The balance between suppressive and cytotoxic responses of the tumor immune microenvironment has been shown to have a direct effect on the final outcome in various human and experimental tumors. Recently, we demonstrated that the oxygen sensor HIF-prolyl hydroxylase 2 (PHD2) plays a detrimental role in tumor cells, stimulating systemic growth and metastasis in mice. In the current study, we show that the conditional ablation of PHD2 in the hematopoietic system also leads to reduced tumor volume, intriguingly generated by an imbalance between enhanced cell death and improved proliferation of tumor cells. This effect seems to rely on the overall down regulation of pro- as well as anti-tumoral cytokines. Using different genetic approaches, we were able to confine this complex phenotype to the crosstalk of PHD2-deficient myeloid cells and T-lymphocytes. Taken together, our findings reveal a multifaceted role for PHD2 in several hematopoietic lineages during tumor development and might have important implications for the development of tumor therapies in the future.

Introduction

Prolyl hydroxylase domain proteins (PHD 1-4) are enzymes which were originally described to hydroxylate subunits of the hypoxia transcription factors HIF1α and HIF2α, inducing their subsequent ubiquitination under normoxic conditions[1]. With decreased levels of oxygen these enzymes become less active, leading to the stabilization of the HIFα subunits. This in turn leads to the onset of HIF downstream adaptation processes, including angiogenesis, survival and metabolic alteration[2]. Furthermore, a number of non-HIF targets of PHDs have already been identified (reviewed in[3]) and even non-hydroxylase activities of PHDs were recently revealed[4, 5]. Our research group previously demonstrated that loss of PHD2 in different tumor cell lines leads to the transformation of TGFβ from a tumor promoter to a tumor suppressor, which resulted in a profound reduction in tumor growth[6, 7]. Contrary to our results, Chan and colleagues have revealed that loss of PHD2 in different human cell lines can lead to faster tumor growth, which they attributed to PHD2-mediated angiogenesis and bone marrow cell recruitment[4]. This highlights the important but also controversial role which PHD2 can assume in different tumor models and settings.

In addition to neoplastic cells, the tumor microenvironment (TME), including stromal cells, endothelial and immune cells play an important role during tumor growth[8]. The latter cells typically originate from the bone marrow[9]. Different immune cells can be found in different amounts depending on the tumor origin and disease-stage. Although it has been well established that the immune system can induce an anti-tumor response[10-12], we now know that this system can be manipulated by tumor cells to do the opposite[13, 14]. Indeed, tumor-associated immune cells have been shown to promote tumor cell proliferation, angiogenesis, metastasis and immune escape[15].

In the present study, we investigated the direct effect of PHD2 gene-targeting in the hematopoietic system in relation to tumor development. We found reduced tumor growth in a recently described conditional PHD2 deficient mouse line which targets the entire

hematopoietic system[16, 17]. In order to unravel the background of this complex phenotype we focused on lineage specific PHD2-deficient mice (myeloid, T-cell and B-cell) and discovered that loss of PHD2 in both - myeloid and T-cells - is necessary and sufficient to reduce tumor growth.

Materials and Methods

Mice

All mice were housed at the Experimental Centre at the University of Technology, Dresden (Medical Faculty, University Hospital Carl-Gustav Carus) and kept under specific pathogen-free conditions. The characterization of the CD68:cre-PHD2$^{f/f}$ and CD68:cre-PHD2/HIF1α$^{ff/ff}$ mouse line has recently been published[16, 17]. For bone marrow transplant experiments lethally irradiated B6.SJL (CD45.1) mice were transplanted with bone marrow from either CD68:cre-PHD2$^{f/f}$ or WT littermates (CD45.2). Uptake of donor bone marrow was verified by FACS using CD45.2/.1 antibodies. For tumor experiments, 1x10^6 Lewis Lung Carcinoma (LLC) cells or 5 x10^5 B16BL6 melanoma cells were injected subcutaneously into both flanks of each mouse. Tumor volume (Volume = (Length x Width^2)/2) was measured every second day for 12 consecutive days using a caliper. Mice were then sacrificed and tumors isolated for flow cytometry analysis or frozen section preparation. All animal experiments were in accordance with the facility guidelines on animal welfare and were approved by the Landesdirektion Dresden, Germany.

Histology

For necrosis analysis, tumors were placed in 4% formaldehyde at 4°C overnight, dehydrated, embedded in paraffin and cut. Sections were rehydrated and subjected to hematoxylin and eosin. For cryo-sections, samples were embedded in OCT, frozen, cut and stored at -20°C. For immunofluorescence analysis sections were first fixed for 10min in cold acetone and blocked with 5% goat serum (Vector labs, #VRC-S-1000) in TNT buffer (20mM Tris pH 7.6; 0.9% NaCl, 0.05% Tween in PBS). Slides were then incubated with the following primary antibodies for either 1hr at 37°C or at 4°C overnight in TNT buffer: rat anti-PECAM 1:1000), rat anti-Ki67 (DAKO, 1:50), rabbit cleaved caspase3 (Cell Signaling, 1:150), HIF1α (Cayman, 1:100) and CD11b (eBioscience, 1:75). Primary antibodies were detected with a secondary antibody bound to Alexa-488 or Alexa-594 (Molecular Probes, 1:400) for 30 min at room temperature. Cell nuclei were stained with DAPI (Sigma, 1:5000). Slides were mounted with fluorescent mounting media (Dako). Fluorescent and light microscopy was done with an Axioplan-2 imaging microscope and plan Apochromat lenses (Carl Zeiss). The cameras and acquisition software were either Q Imaging Retiga 2000R and Image pro MC6.0 (fluo) or Axiocam MRc5 and Axiovision (light). Representative images are presented from experiments with similar results. All quantifications were done using the NIH ImageJ software.

Flow cytometry

FACS analysis was performed on LSRII (Becton Dickinson), sorting was done on Aria II (Becton Dickinson). Data was analyzed on Flowjo (Treestar) software. LLC tumors were isolated and single cell suspensions were prepared by manual mincing with a scalpel, followed by enzymatic digestion by Collagenase D 0.3U/ml (Roche), Dispase II 2.6U/ml (Roche), DNase I 0.05 mg/ml (Roche) in DMEM for 40 min at 37°C. Enzymatic activity was quenched by adding DMEM containing 10% FCS, after which mixture was filtered through 0.7μm nylon strainer (Sefar). Cells were then incubated for 40mins on ice with rat gamma

globulin (Jackson Immunoresearch), rat anti mouse CD16/CD32 (93), CD3 (APC, 145-2C11), CD4 (PE, GK1,5), CD8 (Pe-Cy5, ebioH35-17.2 (H35-17,2)), B220 (A700, RA3-6B2), CD45 (Pe-Cy7, 30-F11), F4/80 (PE, BM8), Gr1 (A700, RB6-8C5), CD11b (FITC, M1/70) (all eBioscience), and DAPI (Molecular Probes) to discriminate between viable and dead cells. At least 6x10^5 cells were acquired. Cells were sorted according to the following antibodies: $CD11b^{+}Gr1\text{-}F4/80^{+}$ for TAM populations, and $CD3^{+}CD4^{+}CD8^{-}$ for $CD4^{+}$ T cell population.

Migration assay

Cell culture chambers (insets of 5 μm pore size, Millipore) were used for the migration of the murine macrophage cell line RAW264.7 cells. RAW cells (1×105 cells/well) were placed in the upper chamber of the insert. Opti-MEM medium containing MCP-1 (10 ng/ml; R&D #479-JE) was placed in the lower chamber. All samples were incubated for 2h at 37˚C in a humidified atmosphere with 5% CO2. In the upper chamber, the non-invasive cells were removed by using a cotton swab and the invasive cells were fixed with methanol stained with hematoxylin. Migrated cells on the lower surface of the filter were counted and photographed using a light microscope at ×40 magnification.

Expression analysis

RNA from sorted cells was isolated using NucleoSpin RNA XS (Machery-Nagel). cDNA was synthesized using random primers (Roche) and superScript II (Invitrogen). Expression levels were determined by performing quantitative Real-time PCR using the Maxima SYBR Green QPCR Master Mix (Fermentas) on an iCycler iQ (Biorad). Sequences of primers used are given in Supplemental data.

Statistical analysis

Data and graphs represent mean ± SEM of representative experiments. Statistical significance was calculated as two-tailed by students t-test (GraphPad Prism v5.04), with $p<0.05$ considered statistically significant.

Results

CD68:cre-PHD2$^{f/f}$ mice display decreased tumor growth, at least partially dependent on HIF1α

We have recently shown that conditional deficiency of PHD2 in erythropoietin (Epo)-producing cells can lead to severe but non-lethal erythrocytosis[16]. In addition, these mice (CD68:cre-PHD2$^{f/f}$) exhibited loss of PHD2 in the entire hematopoietic system and a subset of epithelial cells, but not in endothelium or fibroblasts. In order to study tumor development in this mouse line, we inoculated Lewis lung carcinoma (LLC) cells and monitored tumor growth for 12 days. Interestingly, already 6 days after inoculation, CD68:cre-PHD2$^{f/f}$ mice displayed smaller tumor volumes, which led to a significant difference by day 8 (Fig. 1A). Data from our group[6, 7] and others[4, 18] have suggested opposite roles for PHD2 depending on the tumor type. Therefore, we repeated the experiment–with B16BL6 melanoma cells and once again found significantly reduced tumor volume in the same mouse line (Fig. 1B). Because of the linear growth pattern in the first 12 days, all further experiments were conducted using LLC cells.

Since the CD68:cre-PHD2$^{f/f}$ mouse line was found to contain high Epo accompanied with high red blood cell (RBC) levels in circulation, we sought to find out whether this phenotype mediates the observed differences in tumor volume. Therefore, we repeated the tumor experiment in Epo transgenic mice (Epo Tg6), which display comparable hematocrit levels and slightly higher erythropoietin levels than our CD68:cre-PHD2$^{f/f}$ mice[19]. However, tumor growth in Tg6 mice and WT littermates showed no significant difference (Supplementary Fig. S1A), suggesting that the initial difference in tumor volume observed in CD68:cre-PHD2$^{f/f}$ mice is most probably not related to high Epo concentration in circulation.

The main substrate of PHD2 is HIF1α and therefore we performed the tumor experiments again in CD68:cre-PHD2/HIF1α$^{ff/ff}$ mice, CD68:cre-PHD2$^{f/f}$ and WT counterparts. These double deficient mice also display severe erythrocytosis but in contrast to the cKO mice these

mice die around 11 weeks of age due to neurodegeneration[16]. In this setup, tumor growth was followed for only 10 days, since several double deficient mice died shortly after this time point. Interestingly, in contrast to tumors grown in CD68:cre-PHD2$^{f/f}$ mice, tumors in CD68:cre-PHD2/HIF1α$^{ff/ff}$ mice were not significantly different from WT controls and slightly larger than tumors in the PHD2 conditional deficient mice (Fig. 1C). This suggests that HIF1α might be at least partially responsible for the observed phenotype in CD68:cre-PHD2$^{f/f}$ mice. In addition, we stained tumor sections from the latter mice and WT littermates for CD11b cells and HIF1α. Interestingly, and in contrast to WT tumors, we found that the majority of CD11b-positive myeloid cells in the tumors were also positive for HIF1α (Supplementary Fig. S1B).

Loss of PHD2 in hematopoietic cells leads to decreased LLC tumor growth.

In addition to the erythrocytosis phenotype and PHD2 targeting of the entire hematopoietic system, we also found loss of PHD2 in a few other cell types[16]. In order to undoubtedly validate that the reduced tumor growth is a consequence of targeting the hematopoietic system, we conducted bone marrow transplantation experiments. Whole bone marrow (BM) from either CD68:cre-PHD2$^{f/f}$ mice or WT littermates (CD45.2) was transplanted into lethally irradiated B6.SJL (CD45.1) recipients. Sixteen weeks after transplantation (>98% CD45.2 PMNs in periphery of recipients (data not shown)) both groups were inoculated with LLC tumors. Interestingly, tumor growth in recipients that received cKO BM was significantly slower than in WT counterparts (Fig. 1D), similar to the growth retardation in non-transplanted mice. These results strongly suggest that loss of PHD2 in hematopoietic cells alone is sufficient to reduce the tumor volume.

Tumors in CD68:cre-PHD2$^{f/f}$ mice display enhanced cell death but also increased proliferation

Recently, our group has found that PHD2 silencing in different mouse tumor cell lines is accompanied by the reduced proliferation potential of these tumor cells *in vivo*[6]. We were therefore interested to find out whether the slow growing tumors in the CD68:cre-PHD2$^{f/f}$ mice would display a similar phenotype. Surprisingly, we detected a nearly significant increase (p=0.07) in proliferating Ki67^{+} cells in the smaller tumors isolated from CD68:cre-PHD2$^{f/f}$ mice compared with WT mice (Fig. 2A). Due to this discrepancy we investigated overall cell death in the different tumors. Quantitative analysis show twice as much necrosis in tumors grown in CD68:cre-PHD2$^{f/f}$ mice (Fig. 2B), but no significant difference in cleaved-caspase3; an indicator for apoptosis (Supplementary Fig. S1C). Furthermore, we[6] and others[4] have shown that silencing of PHD2 in tumor cells leads to induced vessel density, albeit in our earlier studies (including LLC cells) it was accompanied with slower growth of the tumor[6]. We therefore investigated the endothelium in these tumors but found no difference in CD31-staining per area (Supplementary Fig. S1D) or in their functionality (data not shown) between both genotypes. Thus, loss of PHD2 in hematopoietic cells leads to reduced tumor growth due to increased necrosis, irrespective of the augmented proliferation rate found in the cKO tumors.

Loss of PHD2 in immune cells does not affect their tumor-homing but leads to an overall reduction in cytokine production

We were interested to figure out whether loss of PHD2 could influence the ability of immune cells to migrate and home to a solid tumor. In a Boyden chamber assay, we first tested the migration ability of the macrophage cell line RAW264.7 in which we silenced PHD2 with two different shPHD2 sequences (Supplementary Fig. S2A)[7]. For this purpose we used monocyte chemotactic protein-1 (MCP1 or CCL2) as a chemo-attractant since it has been shown previously that LLC cells produce this chemokine to attract monocytes/macrophages, while its inhibition can reduce tumor growth in vivo[20]. Interestingly, both PHD2-silenced

RAW cell lines showed significantly reduced migration compared to their controls (Supplementary Fig. S2B). In order to find a link between the reduced tumor growth and loss of PHD2 *in vivo*, we therefore quantified the percentage of immune cells present in the individual tumors of both genotypes at day 12 after inoculation. Despite the difference we found *in vitro*, we detected no significant changes in the percentage of white blood cells ($CD45^+$) between tumors isolated from CD68:cre-$PHD2^{f/f}$ and WT mice (Fig. 3A), nor did we detect any significant difference in tumor associated macrophages (TAMS) ($CD11b^+F4/80^+Gr1^-$), B cells (CD202), cytotoxic ($CD3^+CD8^+$) and helper ($CD3^+CD4^+$) T-cells or myeloid derived suppressor cells (MDSCs) ($CD11b^+Gr1^+$) (Fig. 3B). These findings indicate that loss of PHD2 in immune cells has no influence on the ability of these cells to migrate and home to the solid tumor. Moreover, this result urged us to study the genetic profiles of some of the tumor associated inflammatory cell populations. First of all, we focused on the TAMs since they are the largest inflammatory population in the tumors. Moreover, a great amount of evidence has been collected exposing the importance of these cells in cancer immunology[21, 22]. Unlike the classical anti- (M1) or pro- (M2) tumor gene profile, we found that TAMs isolated from tumors taken out of CD68:cre-$PHD2^{f/f}$ mice showed overall lower expression of pro- as well as anti–inflammatory cytokines compared to controls. Indeed, we didn't only find a significant reduction for TGFβ, iNOS, IL6 and TNFα, the vast majority of tested genes in PHD2 deficient TAMs showed reduced expression (Fig. 3C). Remarkably, this image was also seen in isolated $CD4^+$ T-cells (Fig. 3C), known to interact closely with TAMs in the context of solid tumors and metastasis (reviewed by[23]).

These results imply that reduction of PHD2 in the hematopoietic system of cKO mice leads to a reduction of pro- as well as anti-tumoral genes in TAMs and $CD4^+$ T-cells during tumor formation.

Conditional PHD2 down regulation in distinct immune cell lineages does not affect tumor growth

Our results indicate that loss of PHD2 in the hematopoietic cells can lead to reduced tumor volume, and that TAMs as well as $CD4^+$ T-cells show an overall down-regulation of pro- and anti-tumoral cytokines. We were therefore interested to find out which type of hematopoietic immune cells was responsible for the decreased tumor volume exhibited in the CD68:cre-$PHD2^{f/f}$ mice. We concentrated on the main types of immune cells found in LLC tumors and developed PHD2 conditional deficient mouse lines for each one of them using previously described cre-recombinase mouse lines: LysM:cre for myeloid cells[24], CD4:cre targeting all T-cell populations[25] and CD19:cre for B cells[26]. As shown in Supplementary Fig. S3A-C, reduction of PHD2 was significantly different from WT mice in the appropriate immune cells isolated from tumor bearing transgenic mice.

All mice were inoculated with LLC cells and tumor growth was measured every 2-3 days. In contrast to the results obtained in CD68:cre-$PHD2^{f/f}$ and BM transferred mice, several independent tumor experiments in all three PHD2 conditional KO mice showed no difference in tumor volume compared to their respective WT littermates (Fig. 4A-C). Thus, targeted down regulation of PHD2 solely in myeloid cells, T or B-cells is not responsible for the decrease in tumor volume observed in the cKO mice.

Knock-out of PHD2 in myeloid and T-cells simultaneously reduces tumor growth.

Although data from RNA expression analysis (Fig. 3B) did not indicate an explicit inflammatory response, it exhibited a change in the expression profile of two central immune populations; myeloid cells (TAMs) and T-cells ($CD4^+$ T-cells). Building on these results and data from others groups[27-30], implicating the cross talk between myeloid and T-cells in tumor development, we decided to target both populations simultaneously.

LLC cells were inoculated into LysM/CD4:cre-PHD2$^{f/f}$ and WT littermates, and tumor volume was monitored. Interestingly, several independent experiments showed a significant decrease in tumor growth in the double cre mice compared to their WT littermates (Fig. 5A). To find out whether the tumors from the CD4/LysM:cre-PHD2$^{f/f}$ mice exhibited similar proliferation increase and induction of cell death as found in tumors from the CD68:cre-PHD2$^{f/f}$ mice we stained 12-day old tumors from both genotypes. Interestingly, the tumors from LysM/CD4:cre-PHD2$^{f/f}$ mice displayed a significant increase in proliferation (Ki67 staining) as well as an augmentation of necrotic areas. In these genotypes we distinguished more apoptotic cells, although not significant, but again no difference in vessel density (Supplementary Fig. 4A and B respectively).

Taken together, loss of PHD2 in myeloid cells and T-lymphocytes is necessary and sufficient to delay tumor growth in mice.

Discussion

Studies on the role of PHD2 during tumor development have been mainly limited to cancer cells, whereas not much is known on its effect in tumor associated inflammatory cells. In this report, we demonstrate reduced tumor growth in a mouse line deficient for PHD2 in several cell lineages. Via bone marrow transfer experiments as well as the combination of more confined lineage specific PHD2-deficient mice, we were able to bring back the observed phenotype to both the myeloid and T-cell compartment. Furthermore, we show that the reduced tumor volume is a consequence of opposing changes including the drastic transcriptional down-regulation of numerous pro- as well as anti-tumoral genes in the different immune cells and the induction of cell death together with increased tumor cell proliferation.

Recent work from our research group in an LM8 osteosarcoma and LLC cell line has shown a direct influence of PHD2 on tumor growth in vivo[6]. In that report, PHD2 was silenced in the

tumor cells themselves and resulted in reduced growth. However, this growth retardation was accompanied by slower proliferation of the cells induced by TGFβ and an unexpected increase in vessel density. The current study reveals the importance of PHD2 in the hematopoietic system during the development of primary tumors. For this, we used our recently described CD68:cre-PHD2$^{f/f}$ mouse line that showed loss of PHD2 in the entire hematopoietic system, some subsets of epithelial cells as well as EPO expressing cells in kidney and brain, although not in fibroblasts and endothelial cells[16, 17].

The significantly reduced tumor volume in these conditional PHD2-deficient mice is not only shown in two independent tumor types, we also demonstrate that the growth difference in these erythrocytotic mice was independent of its high EPO levels, in contradiction to previous reports in other tumor settings[31-33]. Furthermore, the fact that LLC tumors are also smaller in mice transplanted with PHD2-deficient BM strongly suggests that the observed phenotype is confined to bone marrow derived cells.

Interestingly, tumor experiments in CD68:cre-PHD2/HIF1α$^{ff/ff}$ mice revealed no significant difference in tumor growth compared to WT littermates or CD68:cre-PHD2$^{f/f}$ mice. This implies that the observed phenotype may in part be driven by HIF1α but probably also by other factors. This could be either via hydroxylase-dependent and/or -independent activities as has been shown for the PHDs in different T-cell contexts before[3, 4, 34-37]. The differential effects that the loss of PHD2 has induced may therefore be a direct link to the unexpected gene profiles we observed in TAMs and CD4^{+} T-cells isolated from day 12 tumors. Indeed, several independent experiments demonstrated an overall down regulation of genes with an anti- (e.g. TNFα, IL1β, iNOS) as well as pro-tumoral (e.g. TGFβ, IL13) signature. These seemingly conflicting results are also reflected in the histology experiments we performed on these tumors. On the one hand, we observed significantly more cell death, but the same tumors also revealed a clear tendency towards more proliferating tumor cells. Taken together, our analysis suggests that in this particular setting, loss of PHD2 seems to generally reduce

the response in TAMs, helper T cells and potentially also other subtypes of tumor associated inflammatory cells (e.g. other T cells and/or MDSCs), which leads to opposing activities and ultimately smaller tumors.

In another line of this study we wanted to unravel which BMDCs were responsible for the observed effects in our broad spectrum PHD2-deficient mouse line. Interestingly, we found that the combined knock-out of PHD2 in myeloid cells and T-lymphocytes was necessary and sufficient to obtain reduced tumor volumes as seen in CD68:cre-PHD2$^{f/f}$ mice. Furthermore, the tumors grown in LysM/CD4:cre-PHD2$^{f/f}$ mice also showed induced cell death combined with a very outspoken induction of Ki67. This apparent crosstalk between these two different classes of immune cells during tumor development is not new. Earlier results conducted by Denardo and colleagues[27] as well as others[38, 39] have emphasized the importance of the interaction between TAMs and CD4$^+$ T lymphocytes in the context of solid tumors and metastasis (reviewed by [23]). Indeed, adaptive immune responses by T lymphocytes can specifically regulate multiple pro-tumor properties of myeloid cells that in turn control many of the "hallmarks" of cancer development[40]. IL4 and IL13 are T_h2 cytokines mainly produced by T-cells that have the ability to entice macrophages to turn into pro-tumoral M2 macrophages by inducing the expression of genes like TGFβ, IL10 and Arg-1[27]. In our study, we found that IL13 and TGFβ were consistently and significantly reduced whereas IL10 and IL4 showed at least a clear tendency to be downregulated. IL6, significantly reduced in the TAMs, is a pro-inflammatory cytokine but has also the potential, in addition to IL10 and TGFβ, to promote the differentiation and polarization of recruited monocytes into M2 macrophages[41]. On the other hand, the T_h1 cytokine IFNγ is able to induce antitumoral responses[42, 43] and recently it was suggested that this could be mediated through the reversion of TAM-induced immunosuppression[44]. Our results did not only show a reduction of this cytokine in the CD4$^+$ tumor associated cells, the TAMs also expressed significantly less iNOS, a vasodilator whose expression is known to be directly regulated by IFNγ[45]. In the

other direction, it was previously shown that TAMs can significantly affect the adaptive immune responses by inducing anergy of naïve T-cells or recruiting and stimulating regulatory T-cells and T_h2 lymphocytes[46]. Thus, our data strongly imply that the availability and recognition of these soluble molecules, directly or indirectly via loss of PHD2 in immune cells, result in important paracrine interactions, which influence growth and viability of the tumor cells.

Taken together, while our results do not implicate a single molecular pathway responsible for the decrease in tumor volume exhibited in CD68:cre-PHD2$^{f/f}$ and LysM/CD4:cre-PHD2$^{f/f}$ mice, they do portray an overall change in different parameters, which lead to the eventual decline in tumor volume. Our findings may also have interesting medical implications as specific PHD2 inhibitors may transform tumors to a less malignant and metastasizing phenotype; not only by the re-induction of the anti-proliferative TGFβ pathway in tumor cells[7] but also, as shown in this study, by disturbing the pro-tumoral activity of the immune system.

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References

1. Epstein AC, Gleadle JM, McNeill LA, Hewitson KS, O'Rourke J, Mole DR, Mukherji M, Metzen E, Wilson MI, Dhanda A, Tian YM, Masson N, et al. C. elegans EGL-9 and mammalian homologs define a family of dioxygenases that regulate HIF by prolyl hydroxylation. Cell 2001;107:43-54.

2. Kaelin WG, Jr., Ratcliffe PJ. Oxygen sensing by metazoans: the central role of the HIF hydroxylase pathway. Mol Cell 2008;30:393-402.

3. Wenger RH, Camenisch G, Stiehl DP, Katschinski DM. HIF prolyl-4-hydroxylase interacting proteins: consequences for drug targeting. Current pharmaceutical design 2009;15:3886-94.

4. Chan DA, Kawahara TLA, Sutphin PD, Chang HY, Chi J-T, Giaccia AJ. Tumor vasculature is regulated by PHD2-mediated angiogenesis and bone marrow-derived cell recruitment. Cancer cell 2009;15:527-38.

5. Xue J, Li X, Jiao S, Wei Y, Wu G, Fang J. Prolyl hydroxylase-3 is down-regulated in colorectal cancer cells and inhibits IKKbeta independent of hydroxylase activity. Gastroenterology 2010;138:606-15.

6. Klotzsche-von Ameln A, Muschter A, Mamlouk S, Kalucka J, Prade I, Franke K, Rezaei M, Poitz DM, Breier G, Wielockx B. Inhibition of HIF prolyl hydroxylase-2 blocks tumor growth in mice through the antiproliferative activity of TGFbeta. Cancer Res 2011;71:3306-16.

7. Klotzsche-von Ameln A, Muschter A, Heimesaat MM, Breier G, Wielockx B. HIF prolyl hydroxylase-2 inhibition diminishes tumor growth through matrix metalloproteinase-induced TGFbeta, activation. Cancer Biol Ther 2012;13:216-23.

8. Kerkar SP, Restifo NP. Cellular constituents of immune escape within the tumor microenvironment. Cancer Res 2012;72:3125-30.

9. Grivennikov SI, Greten FR, Karin M. Immunity, inflammation, and cancer. Cell 2010;140:883-99.

10. Koebel CM, Vermi W, Swann JB, Zerafa N, Rodig SJ, Old LJ, Smyth MJ, Schreiber RD. Adaptive immunity maintains occult cancer in an equilibrium state. Nature 2007;450:903-7.

11. Shankaran V, Ikeda H, Bruce AT, White JM, Swanson PE, Old LJ, Schreiber RD. IFNgamma and lymphocytes prevent primary tumour development and shape tumour immunogenicity. Nature 2001;410:1107-11.

12. Smyth MJ, Swann J, Cretney E, Zerafa N, Yokoyama WM, Hayakawa Y. NKG2D function protects the host from tumor initiation. The Journal of experimental medicine 2005;202:583-8.

13. Schreiber RD, Old LJ, Smyth MJ. Cancer immunoediting: integrating immunity's roles in cancer suppression and promotion. Science 2011;331:1565-70.

14. Balkwill F, Mantovani A. Inflammation and cancer: back to Virchow? Lancet 2001;357:539-45.

15. Biswas SK, Mantovani A. Macrophage plasticity and interaction with lymphocyte subsets: cancer as a paradigm. Nature immunology 2010;11:889-96.

16. Franke K, Kalucka J, Mamlouk S, Singh RP, Muschter A, Weidemann A, Iyengar V, Jahn S, Wieczorek K, Geiger K, Muders M, Sykes AM, et al. HIF-1alpha is a protective factor in conditional PHD2-deficient mice suffering from severe HIF-2alpha-induced excessive erythropoiesis. Blood 2013;121:1436-45.

17. Singh RP, Franke K, Kalucka J, Mamlouk S, Muschter A, Gembarska A, Grinenko T, Willam C, Naumann R, Anastassiadis K, Stewart AF, Bornstein S, et al. HIF-prolyl hydroxylase 2 (PHD2) is a critical regulator of hematopoietic stem cell maintenance during steady-state and stress. Blood 2013.

18. Andersen S, Donnem T, Stenvold H, Al-Saad S, Al-Shibli K, Busund L-T, Bremnes RM. Overexpression of the HIF Hydroxylases PHD1, PHD2, PHD3 and FIH Are Individually and Collectively Unfavorable Prognosticators for NSCLC Survival. PloS one 2011;6:e23847.

19. Ruschitzka FT, Wenger RH, Stallmach T, Quaschning T, de Wit C, Wagner K, Labugger R, Kelm M, Noll G, Rülicke T, Shaw S, Lindberg RL, et al. Nitric oxide prevents cardiovascular disease and

determines survival in polyglobulic mice overexpressing erythropoietin. Proc Natl Acad Sci U S A 2000;97:11609-13.

20. Abangan RS, Jr., Williams CR, Mehrotra M, Duncan JD, Larue AC. MCP1 directs trafficking of hematopoietic stem cell-derived fibroblast precursors in solid tumor. Am J Pathol 2010;176:1914-26.

21. Mantovani A, Sica A. Macrophages, innate immunity and cancer: balance, tolerance, and diversity. Curr Opin Immunol 2010;22:231-7.

22. Pollard JW. Tumour-educated macrophages promote tumour progression and metastasis. Nat Rev Cancer 2004;4:71-8.

23. DeNardo DG, Andreu P, Coussens LM. Interactions between lymphocytes and myeloid cells regulate pro- versus anti-tumor immunity. Cancer metastasis reviews 2010;29:309-16.

24. Clausen BE, Burkhardt C, Reith W, Renkawitz R, F?rster I. Conditional gene targeting in macrophages and granulocytes using LysMcre mice. Transgenic Res 1999;8:265-77.

25. Lee PP, Fitzpatrick DR, Beard C, Jessup HK, Lehar S, Makar KW, Perez-Melgosa M, Sweetser MT, Schlissel MS, Nguyen S, Cherry SR, Tsai JH, et al. A critical role for Dnmt1 and DNA methylation in T cell development, function, and survival. Immunity 2001;15:763-74.

26. Rickert RC, Roes J, Rajewsky K. B lymphocyte-specific, Cre-mediated mutagenesis in mice. Nucleic Acids Res 1997;25:1317-8.

27. DeNardo DG, Barreto JB, Andreu P, Vasquez L, Tawfik D, Kolhatkar N, Coussens LM. CD4(+) T cells regulate pulmonary metastasis of mammary carcinomas by enhancing protumor properties of macrophages. Cancer Cell 2009;16:91-102.

28. Scurr M, Gallimore A, Godkin A. T cell subsets and colorectal cancer: Discerning the good from the bad. Cellular immunology 2012;279:21-4.

29. De Monte L, Reni M, Tassi E, Clavenna D, Papa I, Recalde H, Braga M, Di Carlo V, Doglioni C, Protti MP. Intratumor T helper type 2 cell infiltrate correlates with cancer-associated fibroblast thymic stromal lymphopoietin production and reduced survival in pancreatic cancer. The Journal of experimental medicine 2011;208:469-78.

30. Clark CE, Hingorani SR, Mick R, Combs C, Tuveson DA, Vonderheide RH. Dynamics of the immune reaction to pancreatic cancer from inception to invasion. Cancer research 2007;67:9518-27.

31. Mittelman M, Neumann D, Peled A, Kanter P, Haran-Ghera N. Erythropoietin induces tumor regression and antitumor immune responses in murine myeloma models. Proc Natl Acad Sci U S A 2001;98:5181-6.

32. Okazaki T, Ebihara S, Asada M, Yamanda S, Niu K, Arai H. Erythropoietin promotes the growth of tumors lacking its receptor and decreases survival of tumor-bearing mice by enhancing angiogenesis. Neoplasia 2008;10:932-9.

33. Paragh G, Kumar SM, Rakosy Z, Choi SC, Xu X, Acs G. RNA interference-mediated inhibition of erythropoietin receptor expression suppresses tumor growth and invasiveness in A2780 human ovarian carcinoma cells. Am J Pathol 2009;174:1504-14.

34. Takeda Y, Costa S, Delamarre E, Roncal C, de Oliveira RL, Squadrito ML, Finisguerra V, Deschoemaeker S, Bruy?re Fo, Wenes M, Hamm A, Serneels J, et al. Macrophage skewing by Phd2 haplodeficiency prevents ischaemia by inducing arteriogenesis. Nature 2011;479:122-6.

35. Xue J, Li X, Jiao S, Wei Y, Wu G, Fang J. Prolyl hydroxylase-3 is down-regulated in colorectal cancer cells and inhibits IKKbeta independent of hydroxylase activity. Gastroenterology 2010;138:606-15.

36. Fu J, Taubman MB. Prolyl hydroxylase EGLN3 regulates skeletal myoblast differentiation through an NF-kappaB-dependent pathway. J Biol Chem 2010;285:8927-35.

37. Cummins EP, Berra E, Comerford KM, Ginouves A, Fitzgerald KT, Seeballuck F, Godson C, Nielsen JE, Moynagh P, Pouyssegur J, Taylor CT. Prolyl hydroxylase-1 negatively regulates IkappaB kinase-beta, giving insight into hypoxia-induced NFkappaB activity. Proc Natl Acad Sci U S A 2006;103:18154-9.

38. Wakabayashi O, Yamazaki K, Oizumi S, Hommura F, Kinoshita I, Ogura S, Dosaka-Akita H, Nishimura M. CD4+ T cells in cancer stroma, not CD8+ T cells in cancer cell nests, are associated with favorable prognosis in human non-small cell lung cancers. Cancer science 2003;94:1003-9.

39. Girardi M, Oppenheim D, Glusac EJ, Filler R, Balmain A, Tigelaar RE, Hayday AC. Characterizing the protective component of the alphabeta T cell response to transplantable squamous cell carcinoma. The Journal of investigative dermatology 2004;122:699-706.

40. Hanahan D, Weinberg RA. Hallmarks of cancer: the next generation. Cell 2011;144:646-74.

41. Allavena P, Sica A, Solinas G, Porta C, Mantovani A. The inflammatory micro-environment in tumor progression: the role of tumor-associated macrophages. Critical reviews in oncology/hematology 2008;66:1-9.

42. Street SE, Trapani JA, MacGregor D, Smyth MJ. Suppression of lymphoma and epithelial malignancies effected by interferon gamma. J Exp Med 2002;196:129-34.

43. Windbichler GH, Hausmaninger H, Stummvoll W, Graf AH, Kainz C, Lahodny J, Denison U, Muller-Holzner E, Marth C. Interferon-gamma in the first-line therapy of ovarian cancer: a randomized phase III trial. Br J Cancer 2000;82:1138-44.

44. Duluc D, Corvaisier M, Blanchard S, Catala L, Descamps P, Gamelin E, Ponsoda S, Delneste Y, Hebbar M, Jeannin P. Interferon-gamma reverses the immunosuppressive and protumoral properties and prevents the generation of human tumor-associated macrophages. Int J Cancer 2009;125:367-73.

45. Mazzoni A, Bronte V, Visintin A, Spitzer JH, Apolloni E, Serafini P, Zanovello P, Segal DM. Myeloid suppressor lines inhibit T cell responses by an NO-dependent mechanism. J Immunol 2002;168:689-95.

46. Solinas G, Germano G, Mantovani A, Allavena P. Tumor-associated macrophages (TAM) as major players of the cancer-related inflammation. J Leukoc Biol 2009;86:1065-73.

Legends

Figure 1. Mice deficient for PHD2 in bone marrow-derived cells lead to decreased tumor volume. (A) LLC or (B) B16B6 cells were inoculated in CD68:cre-PHD2$^{f/f}$ mice and their WT littermates and tumor volume was measure every 2 days (n = 27-28 and n = 13-15 respectively). (C) LLC cells were inoculated into mice deficient for PHD2 (CD68:cre-PHD2$^{f/f}$), deficient for both PHD2 and HIF1α (CD68:cre-PHD2/HIF1α$^{ff/ff}$) and WT littermates (n = 7-21) and measured until day 10. (D) LLC cells were inoculated into WT mice which received either bone marrow from CD68:cre-PHD2$^{f/f}$ mice (cKO BM -> WT) or bone marrow from PHD2$^{f/f}$ mice (WT BM-> WT) (n = 15-16). *P < 0.05; **P < 0.01 and ***P < 0.001.

Figure 2. Tumors in CD68:cre-PHD2$^{f/f}$ mice exhibit higher proliferation as well as increased necrosis. (A) Ki67 staining on tumor sections from WT and CD68:cre-PHD2$^{f/f}$ mice representing proliferative potential of the cells (n = 5). (B) H&E staining on tumor sections from WT and CD68:cre-PHD2$^{f/f}$ mice representing cell death (n = 5). *P < 0.05; Scale bars denote 100μm.

Figure 3. Loss of PHD2 in bone-marrow derived cells does not affect homing ability. Single cell suspensions of day 12 LLC tumors were stained and analyzed via flow cytometry. (A) Flow cytometric analysis of total CD45^{+} cells in LLC tumor from CD68:cre-PHD2$^{f/f}$ and WT mice. Data depicted as mean percentage of total viable cells ± SEM (n = 4). (B) Flow cytometric analysis of individual groups of immune cells: B cells (B220+), CD4 T-cells (CD3+CD4+), CD8 T-cells (CD3+CD8+), TAMs (CD11b+Gr1-F4/80+) and MDSCs (CD11b+Gr1+). Data depicted as mean percentage of total CD45^{+} cells ± SEM (n = 4). (C) Cytokine expression by qPCR of TAMs and CD4+ T-cells sorted via FACS from 12 day LLC tumors. Expression depicted as fold change from WT expression (n = 5-10). *P < 0.05.

Figure 4. PHD2 deficient single-populations of leukocytes do not affect tumor volume in mice. LLC cells were inoculated in (A) myeloid (LysM:cre- $PHD2^{f/f}$), (B) T-cell (CD4:cre- $PHD2^{f/f}$) and (C) B cell (CD19:cre- $PHD2^{f/f}$) PHD2 deficient mice. Tumor volume was measured every 2-3 days (n = 7-12).

Figure 5. Loss of PHD2 in myeloid and T-cells leads to decreased tumor volume. (A) LLC cells were inoculated in double cre mice deficient in PHD2 in both myeloid and T-cell compartments (CD4/LysM:cre- $PHD2^{f/f}$) and WT control mice. Tumor volume was measured every 2 days (n = 7-16) * $P < 0.05$ and ** $P < 0.01$. (B) Ki67 staining (top) and H&E staining (bottom) of LLC tumor frozen sections from WT and CD4/LysM:cre-$PHD2^{f/f}$ mice (n=5 and 7 respectively). Error bars represent ± SEM. Scale bars denote 100μm.

Figure 1

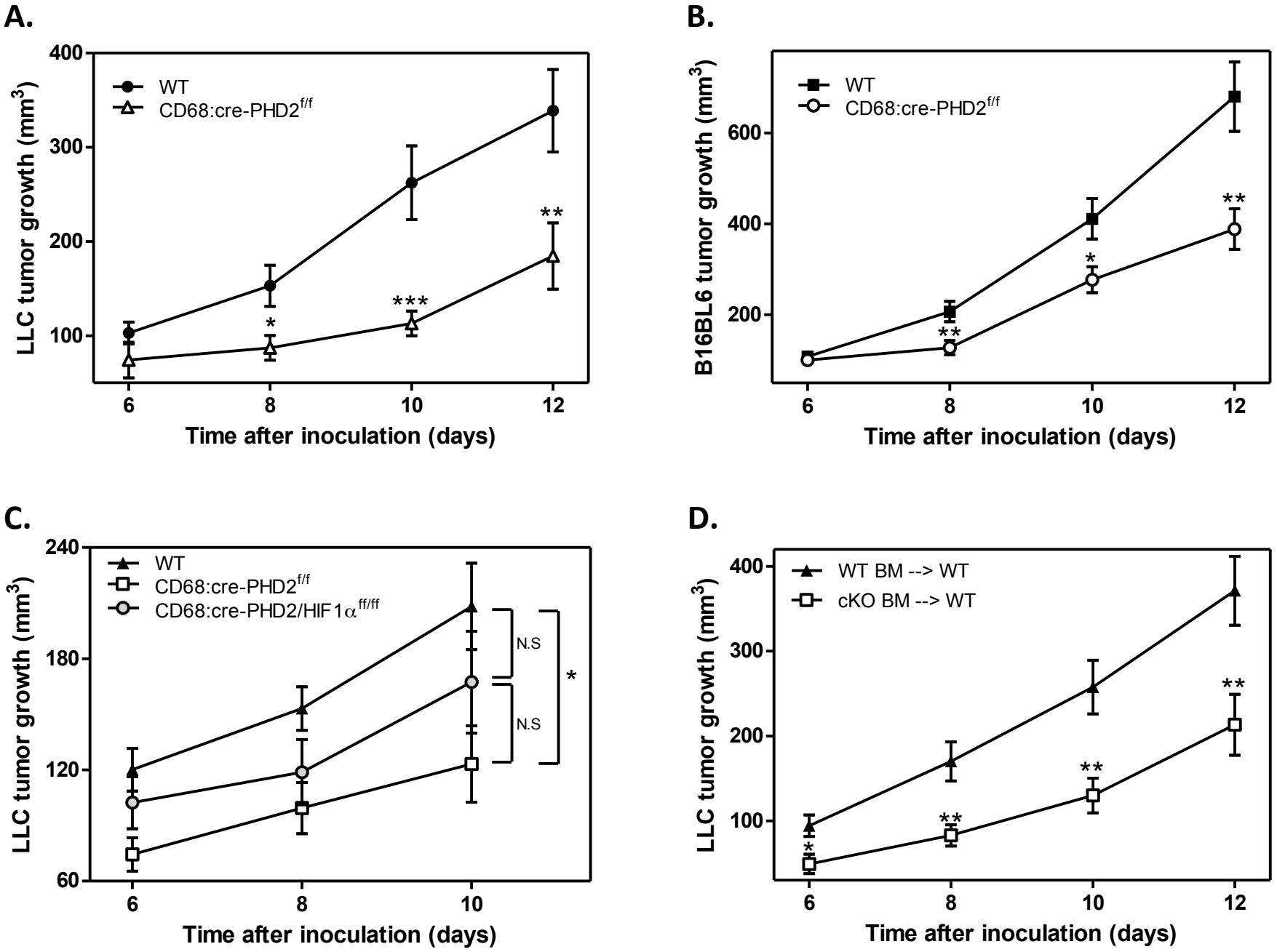

A.
LLC tumor growth (mm3)
WT
CD68:cre-PHD2f/f
Time after inoculation (days)
B.
B16BL6 tumor growth (mm3)
WT
CD68:cre-PHD2f/f
Time after inoculation (days)
C.
LLC tumor growth (mm3)
WT
CD68:cre-PHD2f/f
CD68:cre-PHD2/HIF1αff/ff
N.S
N.S
Time after inoculation (days)
D.
LLC tumor growth (mm3)
WT BM --> WT
cKO BM --> WT
Time after inoculation (days)

Figure 2

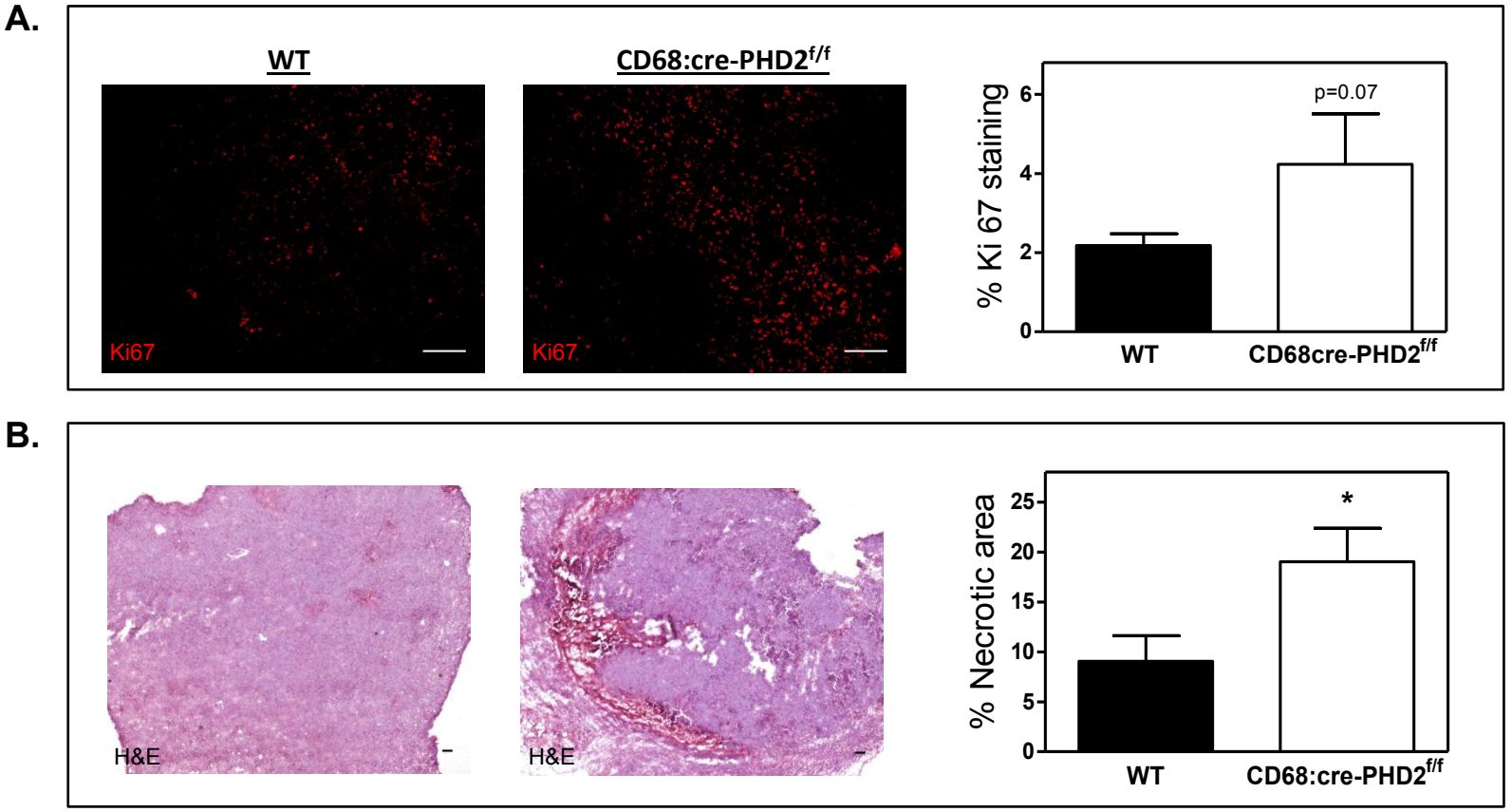

A.
WT
CD68:cre-PHD2f/f
Ki67
Ki67
% Ki 67 staining
p=0.07
0
2
4
6
WT
CD68cre-PHD2f/f
B.
H&E
H&E
% Necrotic area
*
0
5
10
15
20
25
WT
CD68:cre-PHD2f/f

Figure 3

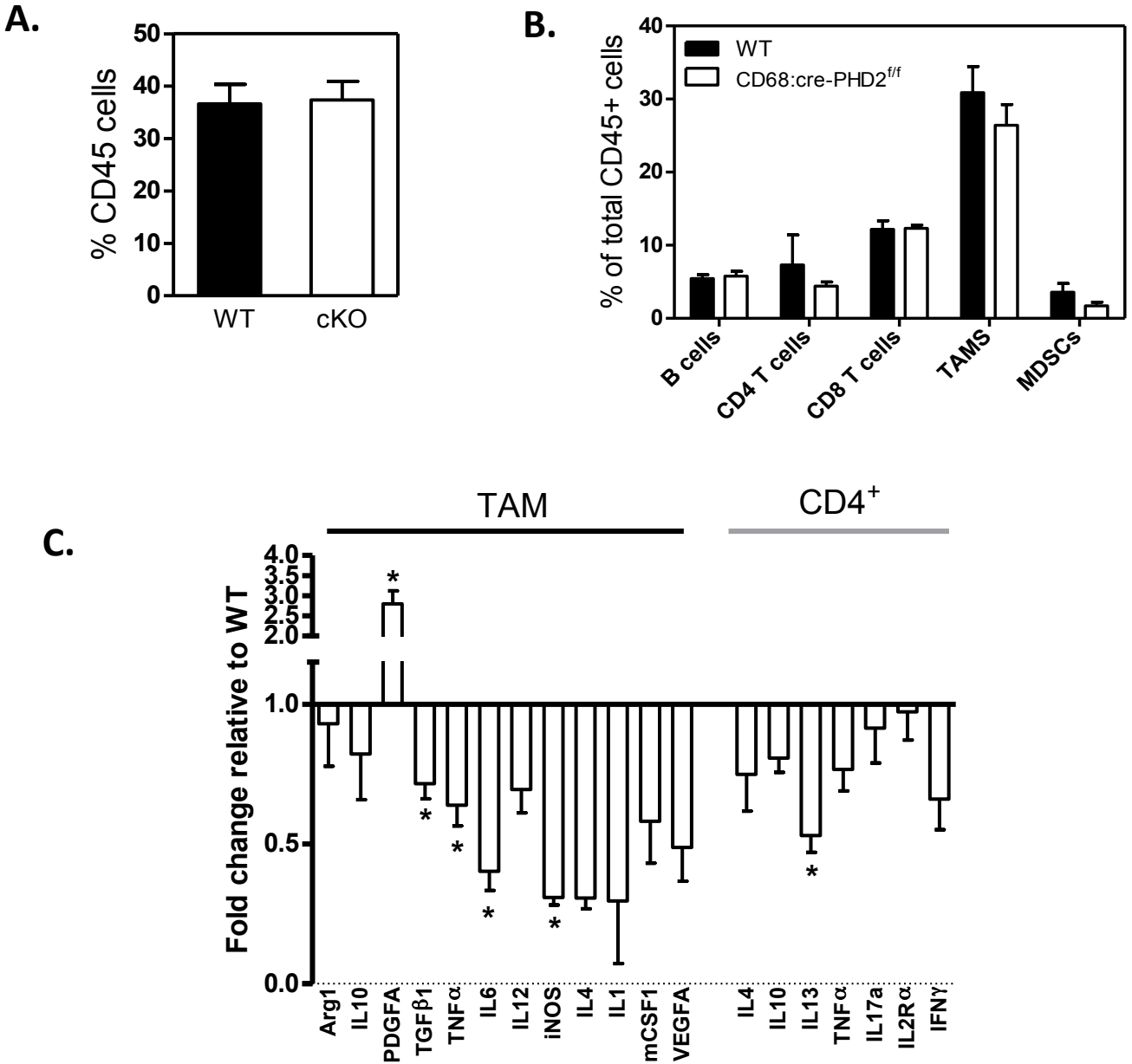

A.
% CD45 cells
50
40
30
20
10
0
WT
cKO
B.
% of total CD45+ cells
40
30
20
10
0
WT
CD68:cre-PHD2f/f
B cells
CD4 T cells
CD8 T cells
TAMS
MDSCs
C.
TAM
CD4+
Fold change relative to WT
4.0
3.5
3.0
2.5
2.0
1.0
0.5
0.0
Arg1
IL10
PDGFA
TGFβ1
TNFα
IL6
IL12
iNOS
IL4
IL1
mCSF1
VEGFA
IL4
IL10
IL13
TNFα
IL17a
IL2Rα
IFNγ

Figure 4

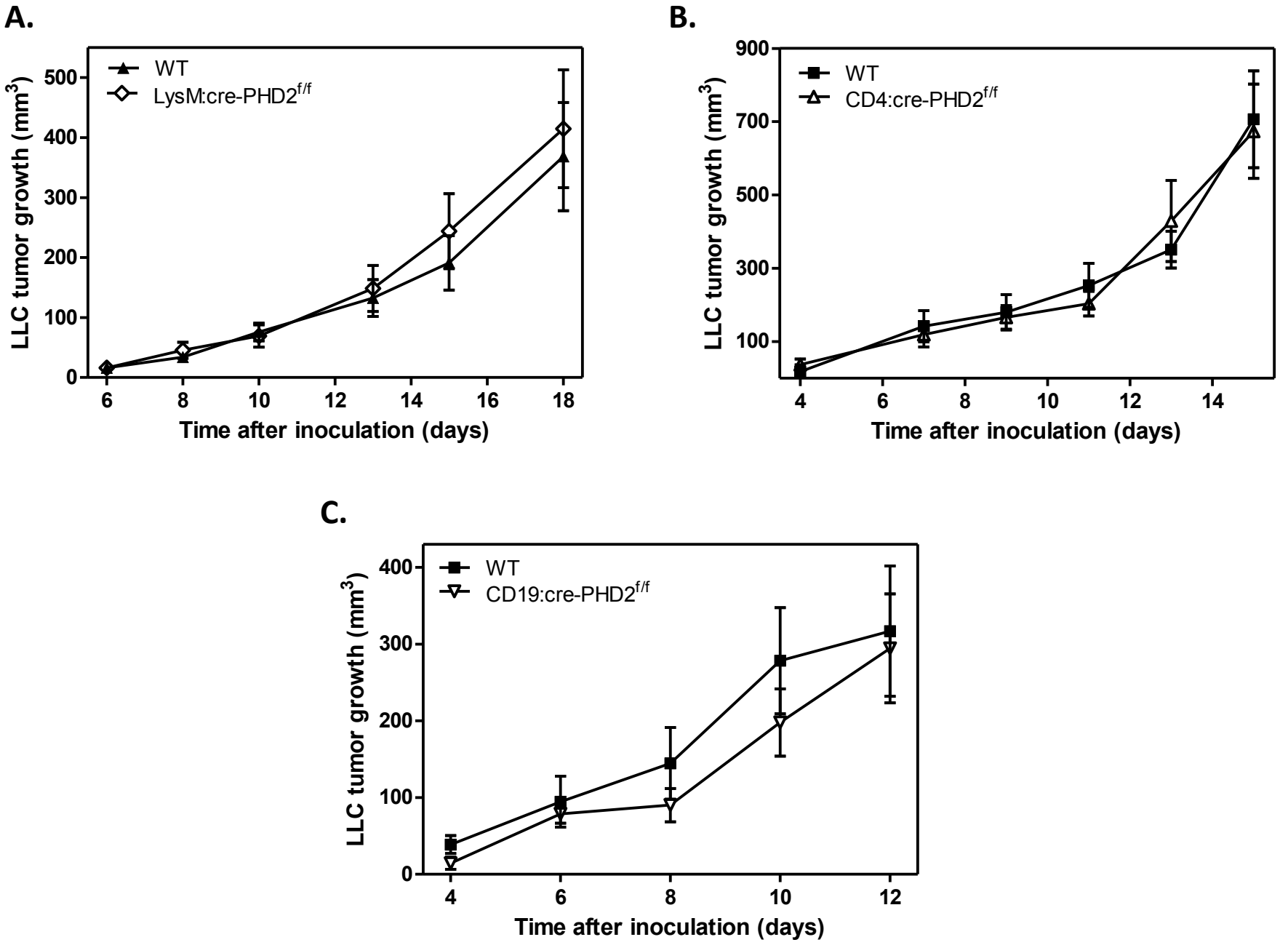
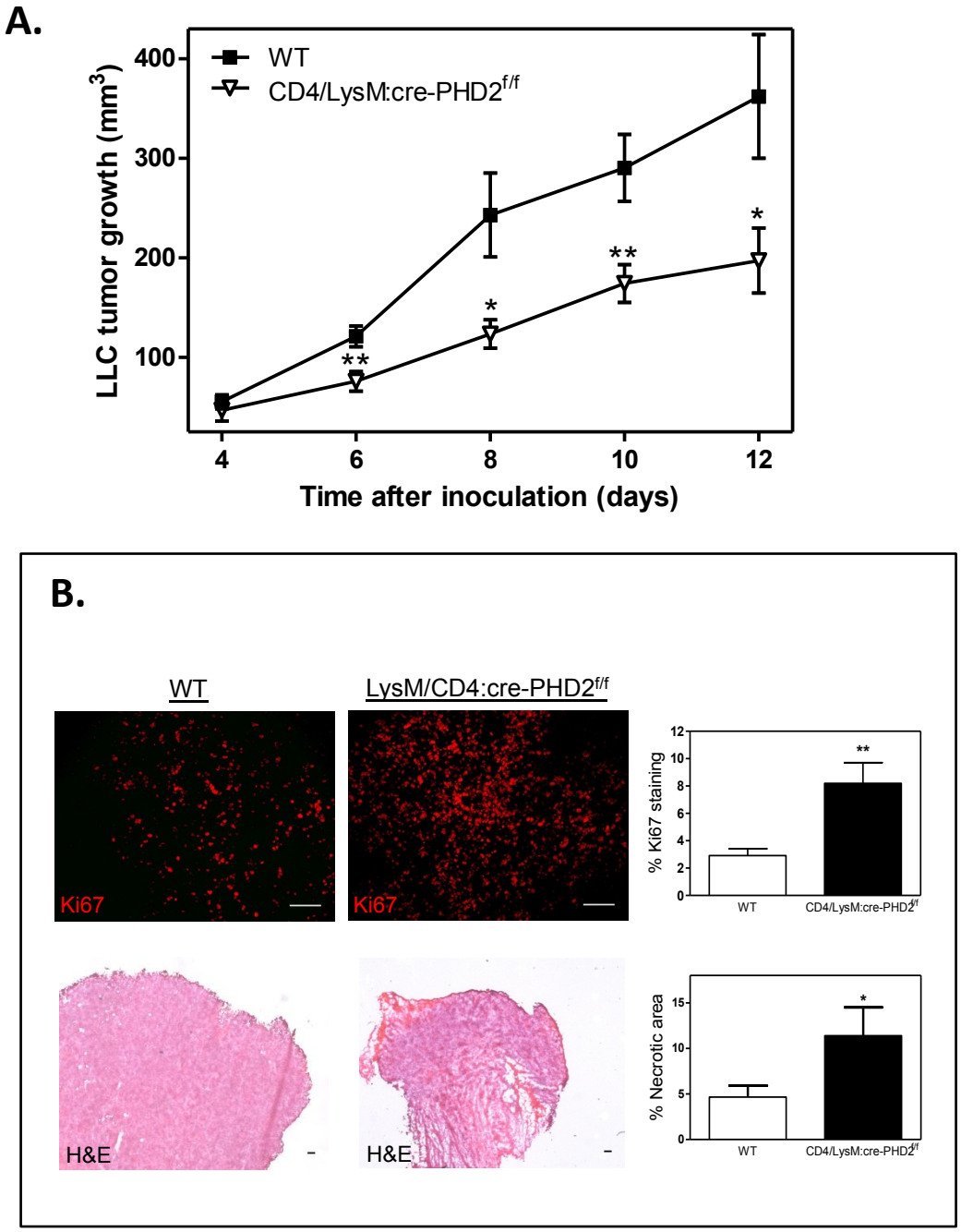
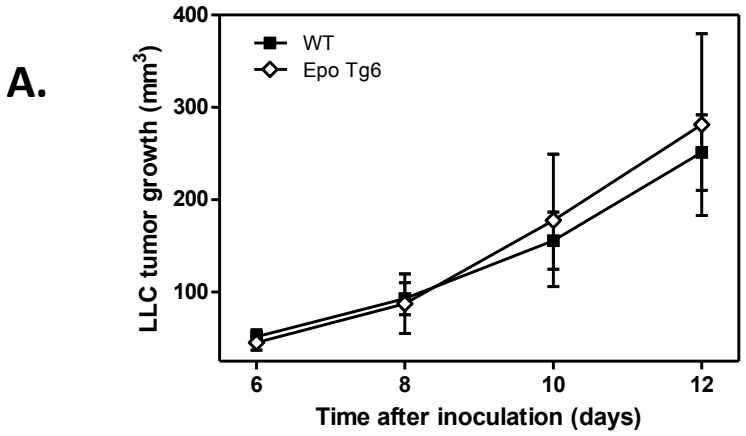
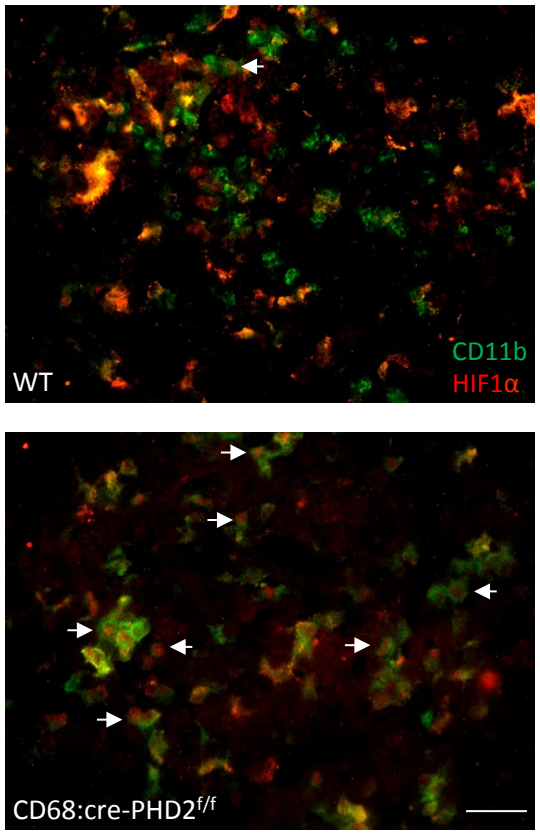

A.
WT
LysM:cre-PHD2f/f
LLC tumor growth (mm3)
Time after inoculation (days)
B.
WT
CD4:cre-PHD2f/f
LLC tumor growth (mm3)
Time after inoculation (days)
C.
WT
CD19:cre-PHD2f/f
LLC tumor growth (mm3)
Time after inoculation (days)

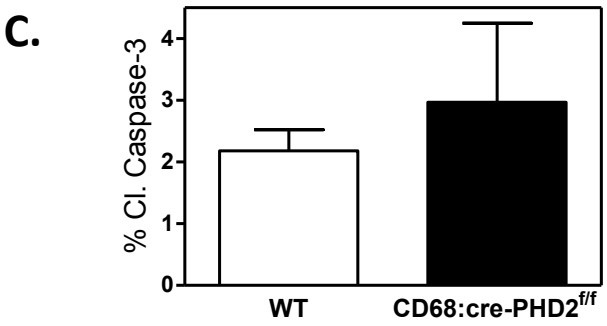
Figure 5

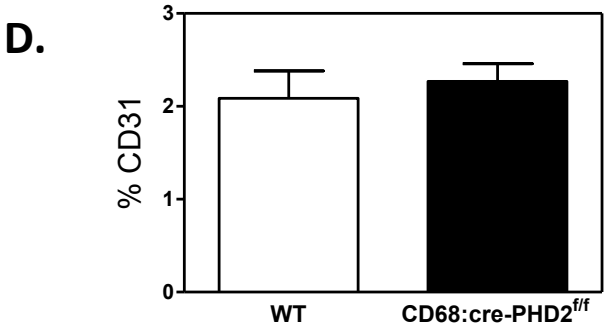

A.
WT
CD4/LysM:cre-PHD2f/f
LLC tumor growth (mm3)
400
300
200
100
4
6
8
10
12
Time after inoculation (days)
**
*
**
*
B.
WT
LysM/CD4:cre-PHD2f/f
Ki67
Ki67
% Ki67 staining
12
10
8
6
4
2
0
**
WT
CD4/LysM:cre-PHD2f/f
H&E
H&E
% Necrotic area
15
10
5
0
*
WT
CD4/LysM:cre-PHD2f/f

Supplementary Figure 1



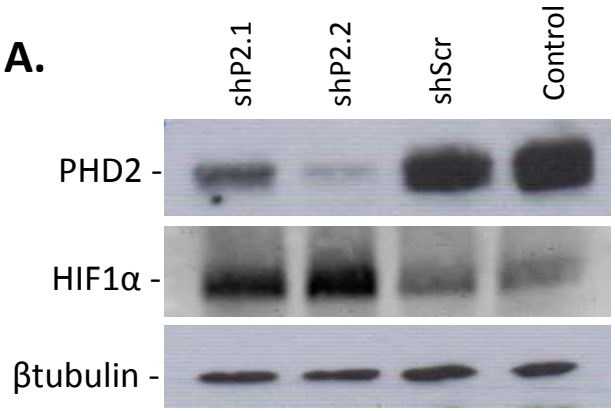


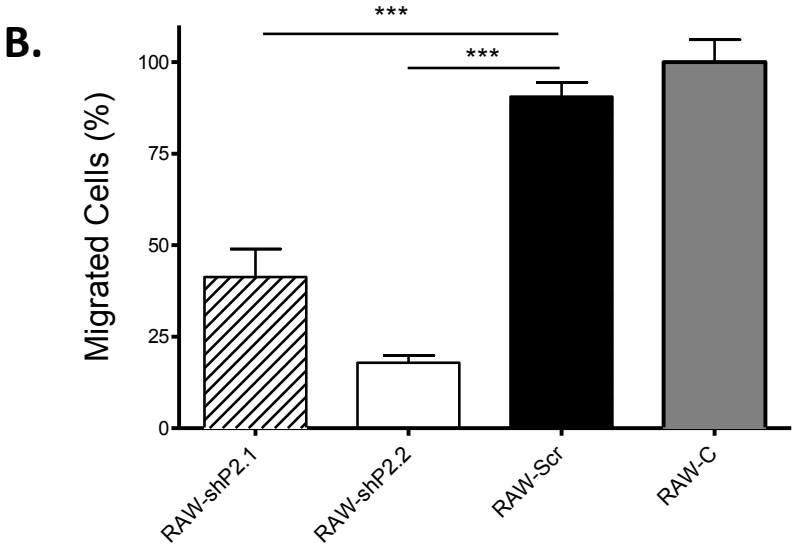




Supplementary figure 1:
(A) Tg 6 mice and WT littermates were inoculated with LLC cells. Tumor volume was measured every 2 days for 12 days (n = 5-9). (B) IHC on tumor sections for HIF1α (Red) and CD11b (Green). (C) Cleaved caspase-3 staining on frozen tumor sections from CD68:cre-PHD2$^{f/f}$ mice and WT littermates representing apoptosis of cells (n = 5-7). (D) CD31 staining of vessels on frozen tumor sections from WT and CD68:cre-PHD2$^{f/f}$ mice (n = 5). Error bars represent ± SEM and Scale bar denotes 25μm.

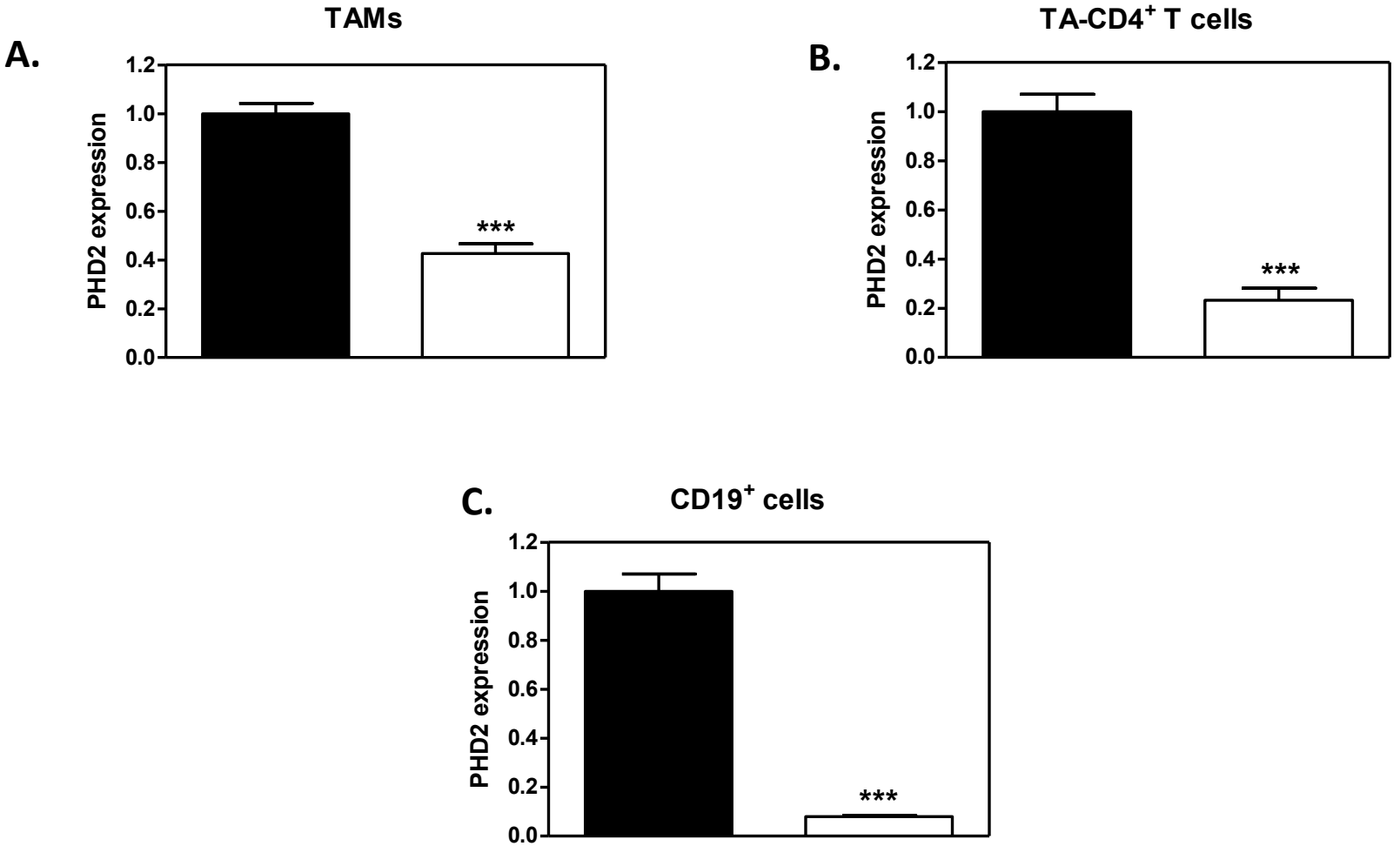
Supplementary Figure 2





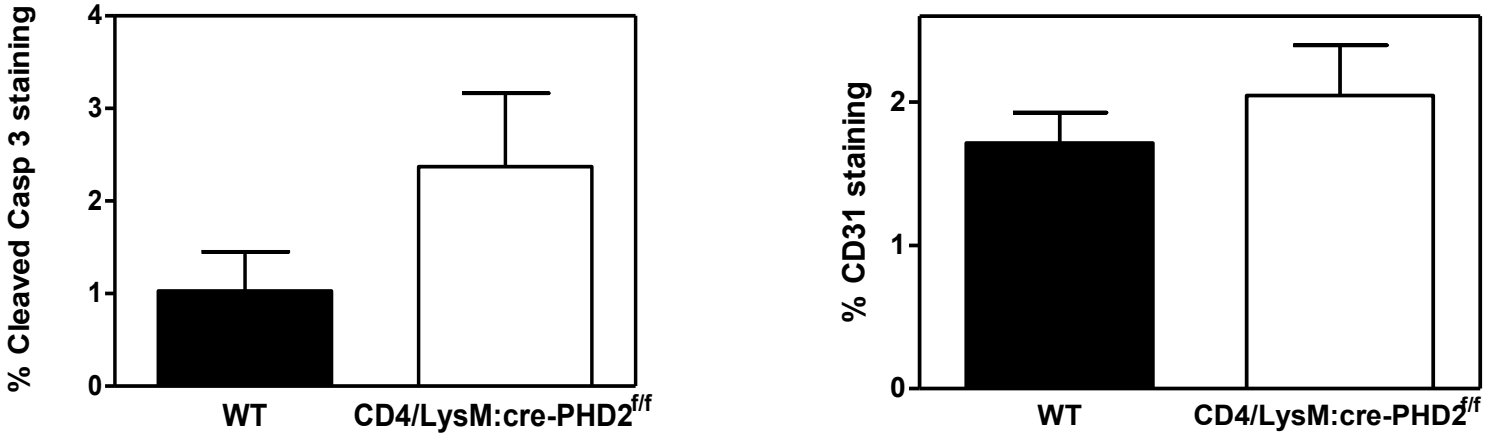
Supplementary figure 2:
(A) RAW cells stably transfected with one of the shRNA constructs against PHD2 (P2.1 and P2.2) or sh scrambled (Scr) and control cells were tested for PHD2, HIF1α and βtubulin protein expression via western blot. (B) Migration assay for RAW shP2.1, shP2.2, shScr and RAW-C cells in a Boyden chamber (n = 5-8). Percentage of cells which migrated relative to the control cells (=100%). Error bars represent ± SEM. ***P < 0.001

Supplementary Figure 3



Supplementary figure 3
(A) Tumor associated macrophages (TAM) ($CD11b^+$, $F4/80^+$, $GR1^-$) and (B) Tumor associated (TA)-$CD4^+$ ($CD3^+CD4^+$) cells were isolated from 12 day old LLC tumors inoculated into CD4:cre-$PHD2^{f/f}$ or LysM:cre-$PHD2^{f/f}$ mice and WT littermates. (C) B-cells ($CD19^+$) were isolated from the lymph nodes from naive CD19:cre-$PHD2^{f/f}$ mice. qPCR analysis of the isolated mRNA content relative to WT. Error bars represent ± SEM. ***$P < 0.001$

Supplementary Figure 4



Supplementary figure 4
(A) Quantification of cleaved caspase-3 on frozen tumor sections isolated from CD4/LysM:cre-PHD2$^{f/f}$ mice and WT littermates representing apoptosis of tumor cells (n = 4-5). (B) % of CD31 staining (vessels) on frozen tumor sections isolated from CD4/ LysM:cre-PHD2$^{f/f}$ mice and WT littermates (n = 4-5). Error bars represent ± SEM.